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(54) Title: COMPOSITIONS AND METHODS FOR siRNA INHIBITION OF HIF-1 ALPHA

(57) Abstract: RNA interference using small interfering RNAs which target HIF-1 alpha mRNA inhibit expression of the HIF-1 alpha gene. As HIF-1 alpha is a transcriptional regulator of VEGF, expression of VEGF is also inhibited. Control of VEGF production through siRNA-mediated down-regulation of HIF-1 alpha can be used to inhibit angiogenesis, in particular in diseases such as diabetic retinopathy, age related macular degeneration and many types of cancer.

COMPOSITIONS AND METHODS FOR siRNA
INHIBITION OF HIF-1 ALPHA

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Cross Reference to Related Application

This application claims the benefit of U.S. provisional patent application serial no. 60/423,262, filed on November 1, 2002.

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Field of the Invention

This invention relates to the regulation of gene expression by siRNA-induced degradation of the transcriptional regulator HIF-1 alpha. In particular, genes in the VEGF mitogenic pathway can be down-regulated.

15

Background of the Invention

Angiogenesis, defined as the growth of new capillary blood vessels, plays a fundamental role in growth and development. In mature humans, the ability to initiate 20 an angiogenic response is present in all tissues, but is held under strict control. A key regulator of angiogenesis is vascular endothelial growth factor ("VEGF"), also called vascular permeability factor ("VPF").

VEGF is expressed in abnormally high levels in certain tissues from diseases characterized by aberrant angiogenesis, such as cancers, diabetic retinopathy, 25 psoriasis, age-related macular degeneration, rheumatoid arthritis and other inflammatory diseases. Therefore, agents which selectively decrease the VEGF levels in these tissues can be used to treat cancer and other angiogenic diseases.

Hypoxia-inducible factor 1 (HIF-1) is a heterodimeric basic-helix-loop-helix-PAS transcription factor consisting of HIF-1 alpha and HIF-1 beta subunits. HIF-1 30 alpha expression and HIF-1 transcriptional activity increase exponentially as cellular oxygen concentration is decreased. Several dozen target genes that are transactivated by HIF-1 have been identified, including those encoding erythropoietin, glucose transporters, glycolytic enzymes, and VEGF. Semenza GL (1999), *Ann. Rev. Cell. Dev. Biol.* 15: 551-578.

Loss of p53 in tumor cells enhances HIF-1 alpha levels and augments HIF-1-dependent transcriptional activation of VEGF in response to hypoxia. Forced expression of HIF-1 alpha in p53-expressing tumor cells increases hypoxia-induced VEGF expression and augments neovascularization and growth of tumor xenografts.

5 These results indicate that amplification of normal HIF-1-dependent responses to hypoxia via loss of p53 function contributes to the angiogenic switch during tumorigenesis. Ravi R. et al. (2000), *Genes Dev.* 14: 34-44.

RNA interference ("RNAi") is a method of post-transcriptional gene regulation that is conserved throughout many eukaryotic organisms. RNAi is induced

10 by short (*i.e.*, <30 nucleotide) double stranded RNA ("dsRNA") molecules which are present in the cell (Fire A et al. (1998), *Nature* 391: 806-811). These short dsRNA molecules, called "short interfering RNA" or "siRNA," cause the destruction of messenger RNAs ("mRNAs") which share sequence homology with the siRNA to within one nucleotide resolution (Elbashir SM et al. (2001), *Genes Dev.* 15: 188-200).

15 It is believed that the siRNA and the targeted mRNA bind to an RNA-induced silencing complex ("RISC"), which cleaves the targeted mRNA. The siRNA is apparently recycled much like a multiple-turnover enzyme, with 1 siRNA molecule capable of inducing cleavage of approximately 1000 mRNA molecules. siRNA-mediated RNAi is therefore more effective than other currently available technologies

20 for inhibiting expression of a target gene.

Elbashir SM et al. (2001), *supra*, has shown that synthetic siRNA of 21 and 22 nucleotides in length, and which have short 3' overhangs, can induce RNAi of target mRNA in a Drosophila cell lysate. Cultured mammalian cells also exhibit RNAi with synthetic siRNA (Elbashir SM et al. (2001) *Nature*, 411: 494-498), and RNAi induced

25 by synthetic siRNA has recently been shown in living mice (McCaffrey AP et al. (2002), *Nature*, 418: 38-39; Xia H et al. (2002), *Nat. Biotech.* 20: 1006-1010). The therapeutic potential of siRNA-mediated RNAi has been demonstrated by several recent *in vitro* studies, including the siRNA-directed inhibition of HIV-1 infection (Novina CD et al. (2002), *Nat. Med.* 8: 681-686) and reduction of neurotoxic

30 polyglutamine disease protein expression (Xia H et al. (2002), *supra*). Therapeutic RNAi has also been demonstrated in human cancer cells by Alan Gewirtz, as described in published U.S. patent application US 2002/0173478.

It has now been found that siRNA-induced RNAi of HIF-1 alpha results in the destruction of HIF-1 alpha mRNA, with a concomitant reduction in VEGF expression and inhibition of angiogenesis.

5

Summary of the Invention

The present invention is directed to siRNAs which specifically target and cause RNAi-induced degradation of mRNA from the human HIF-1 alpha gene. The siRNA compounds and compositions of the invention are used to treat cancerous 10 tumors and other angiogenic diseases and non-pathogenic conditions in which VEGF is overexpressed in tissues in or near the area of neovascularization.

Thus, the invention provides siRNA, and pharmaceutical compositions thereof, which target HIF-1 alpha mRNA and induce RNAi-mediated degradation of the targeted mRNA.

15 The invention further provides a method of inhibiting expression of HIF-1 alpha, comprising administering to a subject an effective amount of an siRNA targeted to HIF-1 alpha mRNA, such that the HIF-1 alpha mRNA is degraded.

20 The invention further provides a method of inhibiting angiogenesis, comprising administering an effective amount of an siRNA targeted to HIF-1 alpha mRNA to a subject, such that the HIF-1 alpha mRNA is degraded and the expression of VEGF is inhibited.

25 The invention further provides a method of treating an angiogenic disease, comprising administering an effective amount of an siRNA targeted to HIF-1 alpha mRNA to a subject, such that the HIF-1 alpha mRNA is degraded and the expression of VEGF is inhibited.

Brief Description of the Drawings

FIG. 1 is a histogram of VEGF concentration, as measured by VEGF ELISA 30 at OD₄₅₀ nanometers, in non-hypoxic (“-”) cultured HEK-293 cells treated with no siRNA (“no”), and in hypoxic (“+”) cultured HEK-293 cells treated with: no siRNA (“no”); nonspecific siRNA (“EGFP”); or with twenty separate siRNAs targeting human HIF-1 alpha mRNA (“hHIF1#1-20”).

FIG. 2 is a histogram showing cytotoxicity in non-hypoxic (“-”) cultured HEK-293 cells treated with no siRNA (“no”), and in hypoxic (“+”) cultured HEK-293 cells treated with: no siRNA (“no”); nonspecific siRNA (“EGFP”); or with twenty separate siRNAs targeting human HIF-1 alpha mRNA (“hHIF1#1-20”).

5 **FIG. 3** is a histogram showing the area of choroidal neovascularization in mm², in eyes from control mice (“control”) and mice treated with anti-HIF-1 alpha siRNA (“HIF-1 siRNA”).

Detailed Description of the Invention

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Compositions and methods comprising siRNA targeted to HIF-1 alpha mRNA are advantageously used to inhibit angiogenesis, in particular for the treatment of angiogenic diseases. The siRNA of the invention causes RNAi-mediated destruction of the HIF-1 alpha mRNA. HIF-1 alpha is a transcriptional regulator of VEGF, and 15 the reduction in HIF-1 alpha mRNA caused by the siRNA of the invention is correlated with a reduction in VEGF production. Because VEGF is required for initiating and maintaining angiogenesis, the siRNA-mediated destruction of HIF-1 alpha slows, stops or reverses the angiogenic process.

As used herein, siRNA which is “targeted to the HIF-1 alpha mRNA” means 20 siRNA in which a first strand of the duplex has the same nucleotide sequence as a portion of the HIF-1 mRNA sequence. It is understood that the second strand of the siRNA duplex is complementary to both the first strand of the siRNA duplex and to the same portion of the HIF-1 alpha mRNA.

The invention therefore provides isolated siRNA comprising short double-stranded RNA from about 17 nucleotides to about 29 nucleotides in length, preferably 25 from about 19 to about 25 nucleotides in length, that are targeted to the target mRNA. The siRNA comprise a sense RNA strand and a complementary antisense RNA strand annealed together by standard Watson-Crick base-pairing interactions (hereinafter “base-paired”). As is described in more detail below, the sense strand comprises a 30 nucleic acid sequence which is substantially identical to a target sequence contained within the target mRNA.

As used herein, a nucleic acid sequence “substantially identical” to a target sequence contained within the target mRNA is a nucleic acid sequence which is

identical to the target sequence, or which differs from the target sequence by one or more nucleotides. Sense strands of the invention which comprise nucleic acid sequences substantially identical to a target sequence are characterized in that siRNA comprising such sense strands induce RNAi-mediated degradation of mRNA containing the target sequence. For example, an siRNA of the invention can comprise a sense strand comprise nucleic acid sequences which differ from a target sequence by one, two or three or more nucleotides, as long as RNAi-mediated degradation of the target mRNA is induced by the siRNA.

The sense and antisense strands of the present siRNA can comprise two complementary, single-stranded RNA molecules or can comprise a single molecule in which two complementary portions are base-paired and are covalently linked by a single-stranded "hairpin" area. Without wishing to be bound by any theory, it is believed that the hairpin area of the latter type of siRNA molecule is cleaved intracellularly by the "Dicer" protein (or its equivalent) to form an siRNA of two individual base-paired RNA molecules (see Tuschl, T. (2002), *supra*). As described below, the siRNA can also contain alterations, substitutions or modifications of one or more ribonucleotide bases. For example, the present siRNA can be altered, substituted or modified to contain one or more deoxyribonucleotide bases.

As used herein, "isolated" means altered or removed from the natural state through human intervention. For example, an siRNA naturally present in a living animal is not "isolated," but a synthetic siRNA, or an siRNA partially or completely separated from the coexisting materials of its natural state is "isolated." An isolated siRNA can exist in substantially purified form, or can exist in a non-native environment such as, for example, a cell into which the siRNA has been delivered.

As used herein, "target mRNA" means human HIF-1 alpha mRNA, mutant or alternative splice forms of human HIF-1 alpha mRNA, or mRNA from cognate HIF-1 alpha genes. A cDNA sequence corresponding to a human HIF-1 alpha mRNA sequence is given in SEQ ID NO: 1.

Splice variants of human HIF-1 alpha are known, including HIF-1 alpha transcript variants 1 (SEQ ID NO: 2) and 2 (SEQ ID NO: 3), as described in GenBank record accession nos. NM_001530 and NM_181054, the entire disclosures of which are herein incorporated by reference. The mRNA transcribed from the human HIF-1

alpha gene can be analyzed for further alternative splice forms using techniques well-known in the art. Such techniques include reverse transcription-polymerase chain reaction (RT-PCR), northern blotting and *in-situ* hybridization. Techniques for analyzing mRNA sequences are described, for example, in Busting SA (2000), *J. Mol. Endocrinol.* 25: 169-193, the entire disclosure of which is herein incorporated by reference. Representative techniques for identifying alternatively spliced mRNAs are also described below.

For example, databases that contain nucleotide sequences related to a given disease gene can be used to identify alternatively spliced mRNA. Such databases 10 include GenBank, Embase, and the Cancer Genome Anatomy Project (CGAP) database. The CGAP database, for example, contains expressed sequence tags (ESTs) from various types of human cancers. An mRNA or gene sequence from the HIF-1 alpha gene can be used to query such a database to determine whether ESTs representing alternatively spliced mRNAs have been found for these genes.

15 A technique called "RNase protection" can also be used to identify alternatively spliced HIF-1 alpha mRNA. RNase protection involves translation of a gene sequence into synthetic RNA, which is hybridized to RNA derived from other cells; for example, cells from tissue at or near the site of neovascularization. The hybridized RNA is then incubated with enzymes that recognize RNA:RNA hybrid 20 mismatches. Smaller than expected fragments indicate the presence of alternatively spliced mRNAs. The putative alternatively spliced mRNAs can be cloned and sequenced by methods well known to those skilled in the art.

RT-PCR can also be used to identify alternatively spliced HIF-1 alpha mRNA. In RT-PCR, mRNA from a tissue is converted into cDNA by the enzyme reverse 25 transcriptase, using methods well-known to those of ordinary skill in the art. The entire coding sequence of the cDNA is then amplified via PCR using a forward primer located in the 3' untranslated region, and a reverse primer located in the 5' untranslated region. The amplified products can be analyzed for alternative splice forms, for example by comparing the size of the amplified products with the size of 30 the expected product from normally spliced mRNA, *e.g.*, by agarose gel electrophoresis. Any change in the size of the amplified product can indicate alternative splicing.

The mRNA produced from a mutant HIF-1 alpha gene can also be readily identified through the techniques described above for identifying alternative splice forms. As used herein, "mutant" HIF-1 alpha gene or mRNA includes a HIF-1 alpha gene or mRNA which differs in sequence from the HIF-1 alpha mRNA sequences set forth herein. Thus, allelic forms of HIF-1 alpha genes, and the mRNA produced from them, are considered "mutants" for purposes of this invention.

As used herein, a gene or mRNA which is "cognate" to human HIF-1 alpha is a gene or mRNA from another mammalian species which is homologous to human HIF-1 alpha. For example, the cognate HIF-1 alpha mRNA from the rat and mouse are described in GenBank record accession nos. NM_024359 and NM_010431, respectively, the entire disclosure of which is herein incorporated by reference. The rat HIF-1 alpha mRNA sequence is given in SEQ ID NO: 4, and the mouse HIF-1 alpha mRNA sequence is given in SEQ ID NO: 5.

It is understood that human HIF-1 alpha mRNA may contain target sequences in common with their respective alternative splice forms, cognates or mutants. A single siRNA comprising such a common targeting sequence can therefore induce RNAi-mediated degradation of different RNA types which contain the common targeting sequence.

The siRNA of the invention can comprise partially purified RNA, substantially pure RNA, synthetic RNA, or recombinantly produced RNA, as well as altered RNA that differs from naturally-occurring RNA by the addition, deletion, substitution and/or alteration of one or more nucleotides. Such alterations can include addition of non-nucleotide material, such as to the end(s) of the siRNA or to one or more internal nucleotides of the siRNA, or modifications that make the siRNA resistant to nuclease digestion, or the substitution of one or more nucleotides in the siRNA with deoxyribonucleotides.

One or both strands of the siRNA of the invention can also comprise a 3' overhang. As used herein, a "3' overhang" refers to at least one unpaired nucleotide extending from the 3'-end of a duplexed RNA strand.

Thus in one embodiment, the siRNA of the invention comprises at least one 3' overhang of from 1 to about 6 nucleotides (which includes ribonucleotides or deoxyribonucleotides) in length, preferably from 1 to about 5 nucleotides in length,

more preferably from 1 to about 4 nucleotides in length, and particularly preferably from about 2 to about 4 nucleotides in length.

In the embodiment in which both strands of the siRNA molecule comprise a 3' overhang, the length of the overhangs can be the same or different for each strand. In 5 a most preferred embodiment, the 3' overhang is present on both strands of the siRNA, and is 2 nucleotides in length. For example, each strand of the siRNA of the invention can comprise 3' overhangs of dithymidylic acid ("TT") or diuridylic acid ("uu").

In order to enhance the stability of the present siRNA, the 3' overhangs can be 10 also stabilized against degradation. In one embodiment, the overhangs are stabilized by including purine nucleotides, such as adenosine or guanosine nucleotides. Alternatively, substitution of pyrimidine nucleotides by modified analogues, *e.g.*, substitution of uridine nucleotides in the 3' overhangs with 2'-deoxythymidine, is tolerated and does not affect the efficiency of RNAi degradation. In particular, the 15 absence of a 2' hydroxyl in the 2'-deoxythymidine significantly enhances the nuclease resistance of the 3' overhang in tissue culture medium.

In certain embodiments, the siRNA of the invention comprises the sequence AA(N19)TT or NA(N21), where N is any nucleotide. These siRNA comprise approximately 30-70% G/C, and preferably comprise approximately 50% G/C. The 20 sequence of the sense siRNA strand corresponds to (N19)TT or N21 (*i.e.*, positions 3 to 23), respectively. In the latter case, the 3' end of the sense siRNA is converted to TT. The rationale for this sequence conversion is to generate a symmetric duplex with respect to the sequence composition of the sense and antisense strand 3' overhangs. The antisense strand is then synthesized as the complement to positions 1 25 to 21 of the sense strand.

Because position 1 of the 23-nt sense strand in these embodiments is not recognized in a sequence-specific manner by the antisense strand, the 3'-most nucleotide residue of the antisense strand can be chosen deliberately. However, the penultimate nucleotide of the antisense strand (complementary to position 2 of the 23- 30 nt sense strand in either embodiment) is generally complementary to the targeted sequence.

In another embodiment, the siRNA of the invention comprises the sequence NAR(N17)YNN, where R is a purine (e.g., A or G) and Y is a pyrimidine (e.g., C or U/T). The respective 21-nt sense and antisense strands of this embodiment therefore generally begin with a purine nucleotide. Such siRNA can be expressed from pol III 5 expression vectors without a change in targeting site, as expression of RNAs from pol III promoters is only believed to be efficient when the first transcribed nucleotide is a purine.

The siRNA of the invention can be targeted to any stretch of approximately 19-25 contiguous nucleotides in any of the target mRNA sequences (the "target 10 sequence"). Techniques for selecting target sequences for siRNA are given, for example, in Tuschl T et al., "The siRNA User Guide," revised Oct. 11, 2002, the entire disclosure of which is herein incorporated by reference. "The siRNA User Guide" is available on the world wide web at a website maintained by Dr. Thomas Tuschl, Department of Cellular Biochemistry, AG 105, Max-Planck-Institute for 15 Biophysical Chemistry, 37077 Göttingen, Germany, and can be found by accessing the website of the Max Planck Institute and searching with the keyword "siRNA." Thus, the sense strand of the present siRNA comprises a nucleotide sequence identical to any contiguous stretch of about 19 to about 25 nucleotides in the target mRNA.

Generally, a target sequence on the target mRNA can be selected from a given 20 cDNA sequence corresponding to the target mRNA, preferably beginning 50 to 100 nt downstream (*i.e.*, in the 3' direction) from the start codon. The target sequence can, however, be located in the 5' or 3' untranslated regions, or in the region nearby the start codon. A suitable target sequence in the HIF-1 alpha cDNA sequence is:

25 AACTGGACACAGTGTGTTGA SEQ ID NO: 6

Thus, an siRNA of the invention targeting this sequence, and which has 3' UU overhangs (overhangs shown in bold) is:

30 5'- **aacuaacuggacacagugugu uu** -3' SEQ ID NO: 7
3'-**uu uugauugaccugugucacaca-5'** SEQ ID NO: 8

An siRNA of the invention targeting this same sequence, but having 3' TT overhangs on each strand (overhangs shown in bold) is:

5 5'-aacuaacuggacacagugugu**TT**-3' (SEQ ID NO: 9)
 3'-**TT**uugauugaccugugucacaca-5' (SEQ ID NO: 10)

Exemplary HIF-1 alpha target sequences from which siRNA of the invention can be derived include those in Table 1 and those given in SEQ ID NOS: 39-296.

10 Table 1 – HIF-1 Alpha Target Sequences

Target sequence	SEQ ID NO:	target sequence	SEQ ID NO:
AACTAACTGGACACAGTGTGT	11	AAGATAAGTTCTGAACG	27
CGACAAGAAAAAGATAA	12	GATAAGTTCTGAACGTC	28
AAAGATAAGTTCTGAAC	13	CGTCGAAAAGAAAAAGTC	29
AGATAAGTTCTGAACGT	14	AGAAAAGTCTCGAGATG	30
GTTCTGAACGTCGAAAAA	15	AAGTCTCGAGATGCAGC	31
AAGAAAAGTCTCGAGAT	16	GTCTCGAGATGCAGCCA	32
GAAAAGTCTCGAGATGC	17	AGAATCTGAAGTTTTT	33
AGTCTCGAGATGCAGCC	18	TCTGAAGTTTTTATGA	34
GTAAAGAATCTGAAGTT	19	TGTGAGTTCCGCATCTTG	35
GAATCTGAAGTTTTTA	20	ACTTCTGGATGCTGGTG	36
GTTTTTATGAGCTTGC	21	GATGACATGAAAGCACA	37
GGCCTCTGTGATGAGGC	22	GCACAGATGAATTGCTT	38
CTTCTGGATGCTGGTGA	23		
AGCACAGATGAATTGCT	24		
AAATGCTTACACACAGAAATG	25		
GAAAAAGATAAGTTCTG	26		

The siRNA of the invention can be obtained using a number of techniques known to those of skill in the art. For example, the siRNA can be chemically synthesized or recombinantly produced using methods known in the art, such as the *Drosophila in vitro* system described in U.S. published application 2002/0086356 of 5 Tuschl et al., the entire disclosure of which is herein incorporated by reference.

Preferably, the siRNA of the invention are chemically synthesized using appropriately protected ribonucleoside phosphoramidites and a conventional DNA/RNA synthesizer. The siRNA can be synthesized as two separate, complementary RNA molecules, or as a single RNA molecule with two 10 complementary regions. Commercial suppliers of synthetic RNA molecules or synthesis reagents include Proligo (Hamburg, Germany), Dharmacon Research (Lafayette, CO, USA), Pierce Chemical (part of Perbio Science, Rockford, IL, USA), Glen Research (Sterling, VA, USA), ChemGenes (Ashland, MA, USA) and Cruachem (Glasgow, UK).

15 Alternatively, siRNA can also be expressed from recombinant circular or linear DNA plasmids using any suitable promoter. Suitable promoters for expressing siRNA of the invention from a plasmid include, for example, the U6 or H1 RNA pol III promoter sequences and the cytomegalovirus promoter. Selection of other suitable promoters is within the skill in the art. The recombinant plasmids of the 20 invention can also comprise inducible or regulatable promoters for expression of the siRNA in a particular tissue or in a particular intracellular environment.

The siRNA expressed from recombinant plasmids can either be isolated from cultured cell expression systems by standard techniques, or can be expressed intracellularly at or near the area of neovascularization *in vivo*. The use of 25 recombinant plasmids to deliver siRNA of the invention to cells *in vivo* is discussed in more detail below.

The siRNA of the invention can be expressed from a recombinant plasmid either as two separate, complementary RNA molecules, or as a single RNA molecule with two complementary regions.

30 Selection of plasmids suitable for expressing siRNA of the invention, methods for inserting nucleic acid sequences for expressing the siRNA into the plasmid, and methods of delivering the recombinant plasmid to the cells of interest are within the

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skill in the art. See, for example Tuschl, T. (2002), *Nat. Biotechnol.* 20: 446-448; Brummelkamp TR et al. (2002), *Science* 296: 550-553; Miyagishi M et al. (2002), *Nat. Biotechnol.* 20: 497-500; Paddison PJ et al. (2002), *Genes Dev.* 16: 948-958; Lee NS et al. (2002), *Nat. Biotechnol.* 20: 500-505; and Paul CP et al. (2002), *Nat. Biotechnol.* 20: 505-508, the entire disclosures of which are herein incorporated by reference.

For example, a plasmid can comprise a sense RNA strand coding sequence in operable connection with a polyT termination sequence under the control of a human U6 RNA promoter, and an antisense RNA strand coding sequence in operable connection with a polyT termination sequence under the control of a human U6 RNA promoter.

As used herein, "in operable connection with a polyT termination sequence" means that the nucleic acid sequences encoding the sense or antisense strands are immediately adjacent to the polyT termination signal in the 5' direction. During transcription of the sense or antisense sequences from the plasmid, the polyT termination signals act to terminate transcription.

As used herein, "under the control" of a promoter means that the nucleic acid sequences encoding the sense or antisense strands are located 3' of the promoter, so that the promoter can initiate transcription of the sense or antisense coding sequences.

The siRNA of the invention can also be expressed from recombinant viral vectors intracellularly at or near the area of neovascularization *in vivo*. The recombinant viral vectors of the invention comprise sequences encoding the siRNA of the invention and any suitable promoter for expressing the siRNA sequences. Suitable promoters include, for example, the U6 or H1 RNA pol III promoter sequences and the cytomegalovirus promoter. Selection of other suitable promoters is within the skill in the art. The recombinant viral vectors of the invention can also comprise inducible or regulatable promoters for expression of the siRNA in a particular tissue or in a particular intracellular environment. The use of recombinant viral vectors to deliver siRNA of the invention to cells *in vivo* is discussed in more detail below.

The siRNA of the invention can be expressed from a recombinant viral vector either as two separate, complementary nucleic acid molecules, or as a single nucleic acid molecule with two complementary regions.

Any viral vector capable of accepting the coding sequences for the siRNA molecule(s) to be expressed can be used, for example vectors derived from adenovirus (AV); adeno-associated virus (AAV); retroviruses (e.g. lentiviruses (LV), Rhabdoviruses, murine leukemia virus); herpes virus, and the like. The tropism of the viral vectors can also be modified by pseudotyping the vectors with envelope proteins or other surface antigens from other viruses. For example, an AAV vector of the invention can be pseudotyped with surface proteins from vesicular stomatitis virus (VSV), rabies, Ebola, Mokola, and the like.

Selection of recombinant viral vectors suitable for use in the invention, methods for inserting nucleic acid sequences for expressing the siRNA into the vector, and methods of delivering the viral vector to the cells of interest are within the skill in the art. See, for example, Dornburg R (1995), *Gene Therap.* 2: 301-310; Eglitis MA (1988), *Biotechniques* 6: 608-614; Miller AD (1990), *Hum Gene Therap.* 1: 5-14; and Anderson WF (1998), *Nature* 392: 25-30, the entire disclosures of which are herein incorporated by reference.

Preferred viral vectors are those derived from AV and AAV. In a particularly preferred embodiment, the siRNA of the invention is expressed as two separate, complementary single-stranded RNA molecules from a recombinant AAV vector comprising, for example, either the U6 or H1 RNA promoters, or the cytomegalovirus (CMV) promoter.

A suitable AV vector for expressing the siRNA of the invention, a method for constructing the recombinant AV vector, and a method for delivering the vector into target cells, are described in Xia H et al. (2002), *Nat. Biotech.* 20: 1006-1010.

Suitable AAV vectors for expressing the siRNA of the invention, methods for constructing the recombinant AAV vector, and methods for delivering the vectors into target cells are described in Samulski R et al. (1987), *J. Virol.* 61: 3096-3101; Fisher KJ et al. (1996), *J. Virol.*, 70: 520-532; Samulski R et al. (1989), *J. Virol.* 63: 3822-3826; U.S. Pat. No. 5,252,479; U.S. Pat. No. 5,139,941; International Patent

Application No. WO 94/13788; and International Patent Application No. WO 93/24641, the entire disclosures of which are herein incorporated by reference.

The ability of an siRNA containing a given target sequence to cause RNAi-mediated degradation of the target mRNA can be evaluated using standard techniques 5 for measuring the levels of RNA or protein in cells. For example, siRNA of the invention can be delivered to cultured cells, and the levels of target mRNA can be measured by Northern blot or dot blotting techniques, or by quantitative RT-PCR. Alternatively, the levels of HIF-1 alpha protein in the cultured cells can be measured by ELISA or Western blot. A suitable cell culture system for measuring the effect of 10 the present siRNA on target mRNA or protein levels is described in Example 1 below.

The ability of an siRNA to target and cause RNAi-mediated degradation of HIF-1 alpha mRNA can also be evaluated by measuring the levels of VEGF mRNA or protein in cultured cells, as a reduction in HIF-1 alpha expression will also inhibit VEGF expression.

15 For example, 50% confluent 293 human kidney cells can be incubated with culture medium containing an siRNA (optionally complexed to a transfection reagent such as Mirus Transit TKO transfection reagent) for 48 hours, followed by ELISA or mRNA quantification of either HIF-1 alpha or VEGF. Cells incubated with an siRNA not homologous to the HIF-1 alpha target sequence can be used as controls.

20 RNAi-mediated degradation of target mRNA by an siRNA containing a given target sequence can also be evaluated with animal models of neovascularization, such as the retinopathy of prematurity ("ROP") or choroidal neovascularization ("CNV") mouse models. For example, areas of neovascularization in an ROP or CNV mouse can be measured before and after administration of an siRNA. A reduction in the 25 areas of neovascularization in these models upon administration of the siRNA indicates the down-regulation of the target mRNA (see Example 2 below).

As discussed above, the siRNA of the invention target and cause the RNAi-mediated degradation of HIF-1 alpha mRNA, or alternative splice forms, mutants or cognates thereof. Degradation of the target mRNA by the present siRNA reduces the 30 production of a functional gene product from the HIF-1 alpha gene. Thus, the invention provides a method of inhibiting expression of HIF-1 alpha in a subject, comprising administering an effective amount of an siRNA of the invention to the

subject, such that the target mRNA is degraded. In the practice of the present methods, it is understood that more than one siRNA of the invention can be administered simultaneously to the subject.

Without wishing to be bound by any theory, the products of the HIF-1 alpha 5 gene are believed to be involved in the transcriptional regulation of VEGF. VEGF is in turn required for initiating and maintaining angiogenesis. Thus, the invention also provides a method of inhibiting angiogenesis in a subject by the RNAi-mediated degradation of the target mRNA by an siRNA of the invention.

As used herein, a "subject" includes a human being or non-human animal. 10 Preferably, the subject is a human being.

As used herein, an "effective amount" of the siRNA is an amount sufficient to cause RNAi-mediated degradation of the target mRNA, or an amount sufficient to inhibit angiogenesis in a subject.

RNAi-mediated degradation of the target mRNA can be detected by 15 measuring levels of the target mRNA or protein in the cells of a subject, using standard techniques for isolating and quantifying mRNA or protein as described above.

Inhibition of angiogenesis can be evaluated by directly measuring the progress of pathogenic or nonpathogenic angiogenesis in a subject; for example, by observing 20 the size of a neovascularized area before and after treatment with the siRNA of the invention. An inhibition of angiogenesis is indicated if the size of the neovascularized area stays the same or is reduced. Techniques for observing and measuring the size of neovascularized areas in a subject are within the skill in the art; for example, areas of choroid neovascularization can be observed by ophthalmoscopy.

Inhibition of angiogenesis can also be inferred through observing a change or 25 reversal in a pathogenic condition associated with the angiogenesis. For example, in ARMD, a slowing, halting or reversal of vision loss indicates an inhibition of angiogenesis in the choroid. For tumors, a slowing, halting or reversal of tumor growth, or a slowing or halting of tumor metastasis, indicates an inhibition of 30 angiogenesis at or near the tumor site. Inhibition of non-pathogenic angiogenesis can also be inferred from, for example, fat loss or a reduction in cholesterol levels upon administration of the siRNA of the invention.

It is understood that the siRNA of the invention can degrade the target mRNA (and thus inhibit angiogenesis) in substoichiometric amounts. Without wishing to be bound by any theory, it is believed that the siRNA of the invention induces the RISC to degrade of the target mRNA in a catalytic manner. Thus, compared to standard 5 anti-angiogenic therapies, significantly less siRNA needs to be delivered at or near the site of neovascularization to have a therapeutic effect.

One skilled in the art can readily determine an effective amount of the siRNA of the invention to be administered to a given subject, by taking into account factors such as the size and weight of the subject; the extent of the neovascularization or disease 10 penetration; the age, health and sex of the subject; the route of administration; and whether the administration is regional or systemic. Generally, an effective amount of the siRNA of the invention comprises an amount which provides an intercellular concentration at or near the neovascularization site of from about 1 nanomolar (nM) to about 100 nM, preferably from about 2 nM to about 50 nM, more preferably from about 2.5 nM to 15 about 10 nM. It is contemplated that greater or lesser amounts of siRNA can be administered.

The present methods can be used to inhibit angiogenesis which is non-pathogenic; *i.e.*, angiogenesis which results from normal processes in the subject. Examples of non-pathogenic angiogenesis include endometrial neovascularization, 20 and processes involved in the production of fatty tissues or cholesterol. Thus, the invention provides a method for inhibiting non-pathogenic angiogenesis, *e.g.*, for controlling weight or promoting fat loss, for reducing cholesterol levels, or as an abortifacient.

The present methods can also inhibit angiogenesis which is associated with an 25 angiogenic disease; *i.e.*, a disease in which pathogenicity is associated with inappropriate or uncontrolled angiogenesis. For example, most cancerous solid tumors generate an adequate blood supply for themselves by inducing angiogenesis in and around the tumor site. This tumor-induced angiogenesis is often required for tumor growth, and also allows metastatic cells to enter the bloodstream.

30 Other angiogenic diseases include diabetic retinopathy, age-related macular degeneration (ARMD), psoriasis, rheumatoid arthritis and other inflammatory diseases. These diseases are characterized by the destruction of normal tissue by

newly formed blood vessels in the area of neovascularization. For example, in ARMD, the choroid is invaded and destroyed by capillaries. The angiogenesis-driven destruction of the choroid in ARMD eventually leads to partial or full blindness.

Preferably, an siRNA of the invention is used to inhibit the growth or 5 metastasis of solid tumors associated with cancers; for example breast cancer, lung cancer, head and neck cancer, brain cancer, abdominal cancer, colon cancer, colorectal cancer, esophagus cancer, gastrointestinal cancer, glioma, liver cancer, tongue cancer, neuroblastoma, osteosarcoma, ovarian cancer, pancreatic cancer, prostate cancer, retinoblastoma, Wilm's tumor, multiple myeloma; skin cancer (e.g., 10 melanoma), lymphomas and blood cancer.

More preferably, an siRNA of the invention is used to inhibit choroidal neovascularization in age-related macular degeneration.

For treating angiogenic diseases, the siRNA of the invention can administered 15 to a subject in combination with a pharmaceutical agent which is different from the present siRNA. Alternatively, the siRNA of the invention can be administered to a subject in combination with another therapeutic method designed to treat the angiogenic disease. For example, the siRNA of the invention can be administered in combination with therapeutic methods currently employed for treating cancer or preventing tumor metastasis (e.g., radiation therapy, chemotherapy, and surgery). For 20 treating tumors, the siRNA of the invention is preferably administered to a subject in combination with radiation therapy, or in combination with chemotherapeutic agents such as cisplatin, carboplatin, cyclophosphamide, 5-fluorouracil, adriamycin, daunorubicin or tamoxifen.

In the present methods, the present siRNA can be administered to the subject 25 either as naked siRNA, in conjunction with a delivery reagent, or as a recombinant plasmid or viral vector which expresses the siRNA.

Suitable delivery reagents for administration in conjunction with the present siRNA include the Mirus Transit TKO lipophilic reagent; lipofectin; lipofectamine; cellfectin; or polycations (e.g., polylysine), or liposomes. A preferred delivery 30 reagent is a liposome.

Liposomes can aid in the delivery of the siRNA to a particular tissue, such as retinal or tumor tissue, and can also increase the blood half-life of the siRNA.

Liposomes suitable for use in the invention are formed from standard vesicle-forming lipids, which generally include neutral or negatively charged phospholipids and a sterol, such as cholesterol. The selection of lipids is generally guided by consideration of factors such as the desired liposome size and half-life of the 5 liposomes in the blood stream. A variety of methods are known for preparing liposomes, for example as described in Szoka et al. (1980), *Ann. Rev. Biophys. Bioeng.* 9: 467; and U.S. Pat. Nos. 4,235,871, 4,501,728, 4,837,028, and 5,019,369, the entire disclosures of which are herein incorporated by reference.

Preferably, the liposomes encapsulating the present siRNA comprise a ligand 10 molecule that can target the liposome to a particular cell or tissue at or near the site of angiogenesis. Ligands which bind to receptors prevalent in tumor or vascular endothelial cells, such as monoclonal antibodies that bind to tumor antigens or endothelial cell surface antigens, are preferred.

Particularly preferably, the liposomes encapsulating the present siRNA are 15 modified so as to avoid clearance by the mononuclear macrophage and reticuloendothelial systems, for example by having opsonization-inhibition moieties bound to the surface of the structure. In one embodiment, a liposome of the invention can comprise both opsonization-inhibition moieties and a ligand.

Opsonization-inhibiting moieties for use in preparing the liposomes of the 20 invention are typically large hydrophilic polymers that are bound to the liposome membrane. As used herein, an opsonization inhibiting moiety is "bound" to a liposome membrane when it is chemically or physically attached to the membrane, e.g., by the intercalation of a lipid-soluble anchor into the membrane itself, or by binding directly to active groups of membrane lipids. These opsonization-inhibiting 25 hydrophilic polymers form a protective surface layer which significantly decreases the uptake of the liposomes by the macrophage-monocyte system ("MMS") and reticuloendothelial system ("RES"); e.g., as described in U.S. Pat. No. 4,920,016, the entire disclosure of which is herein incorporated by reference. Liposomes modified with opsonization-inhibition moieties thus remain in the circulation much longer than 30 unmodified liposomes. For this reason, such liposomes are sometimes called "stealth" liposomes.

Stealth liposomes are known to accumulate in tissues fed by porous or "leaky" microvasculature. Thus, target tissue characterized by such microvasculature defects, for example solid tumors, will efficiently accumulate these liposomes; *see* Gabizon, et al. (1988), *P.N.A.S., USA*, 18: 6949-53. In addition, the reduced uptake by the RES 5 lowers the toxicity of stealth liposomes by preventing significant accumulation in the liver and spleen. Thus, liposomes of the invention that are modified with opsonization-inhibition moieties can deliver the present siRNA to tumor cells.

Opsonization inhibiting moieties suitable for modifying liposomes are preferably water-soluble polymers with a number-average molecular weight from 10 about 500 to about 40,000 daltons, and more preferably from about 2,000 to about 20,000 daltons. Such polymers include polyethylene glycol (PEG) or polypropylene glycol (PPG) derivatives; e.g., methoxy PEG or PPG, and PEG or PPG stearate; synthetic polymers such as polyacrylamide or poly N-vinyl pyrrolidone; linear, branched, or dendrimeric polyamidoamines; polyacrylic acids; polyalcohols, e.g., 15 polyvinylalcohol and polyxylitol to which carboxylic or amino groups are chemically linked, as well as gangliosides, such as ganglioside GM₁. Copolymers of PEG, methoxy PEG, or methoxy PPG, or derivatives thereof, are also suitable. In addition, the opsonization inhibiting polymer can be a block copolymer of PEG and either a polyamino acid, polysaccharide, polyamidoamine, polyethyleneamine, or 20 polynucleotide. The opsonization inhibiting polymers can also be natural polysaccharides containing amino acids or carboxylic acids, e.g., galacturonic acid, glucuronic acid, mannuronic acid, hyaluronic acid, pectic acid, neuraminic acid, alginic acid, carrageenan; aminated polysaccharides or oligosaccharides (linear or branched); or carboxylated polysaccharides or oligosaccharides, e.g., reacted with 25 derivatives of carbonic acids with resultant linking of carboxylic groups.

Preferably, the opsonization-inhibiting moiety is a PEG, PPG, or derivatives thereof. Liposomes modified with PEG or PEG-derivatives are sometimes called "PEGylated liposomes."

The opsonization inhibiting moiety can be bound to the liposome membrane 30 by any one of numerous well-known techniques. For example, an N-hydroxysuccinimide ester of PEG can be bound to a phosphatidyl-ethanolamine lipid-soluble anchor, and then bound to a membrane. Similarly, a dextran polymer can be

derivatized with a stearylamine lipid-soluble anchor via reductive amination using Na(CN)BH₃ and a solvent mixture such as tetrahydrofuran and water in a 30:12 ratio at 60 °C.

Recombinant plasmids which express siRNA of the invention are discussed above. Such recombinant plasmids can also be administered to a subject directly or in conjunction with a suitable delivery reagent, including the Mirus Transit LT1 lipophilic reagent; lipofectin; lipofectamine; cellfectin; polycations (e.g., polylysine) or liposomes. Recombinant viral vectors which express siRNA of the invention are also discussed above, and methods for delivering such vectors to an area of neovascularization in a subject are within the skill in the art.

The siRNA of the invention can be administered to the subject by any means suitable for delivering the siRNA to the cells of the tissue at or near the area of neovascularization. For example, the siRNA can be administered by gene gun, electroporation, or by other suitable parenteral or enteral administration routes.

Suitable enteral administration routes include oral, rectal, or intranasal delivery.

Suitable parenteral administration routes include intravascular administration (e.g. intravenous bolus injection, intravenous infusion, intra-arterial bolus injection, intra-arterial infusion and catheter instillation into the vasculature); peri- and intra-tissue administration (e.g., peri-tumoral and intra-tumoral injection, intra-retinal injection or subretinal injection); subcutaneous injection or deposition including subcutaneous infusion (such as by osmotic pumps); direct (e.g., topical) application to the area at or near the site of neovascularization, for example by a catheter or other placement device (e.g., a corneal pellet or a suppository, eye-dropper, or an implant comprising a porous, non-porous, or gelatinous material); and inhalation. Suitable placement devices include the ocular implants described in U.S. Pat. Nos. 5,902,598 and 6,375,972, and the biodegradable ocular implants described in U.S. Pat. No 6,331,313, the entire disclosures of which are herein incorporated by reference. Such ocular implants are available from Control Delivery Systems, Inc. (Watertown, MA) and Oculex Pharmaceuticals, Inc. (Sunnyvale, CA).

In a preferred embodiment, injections or infusions of the siRNA are given at or near the site of neovascularization. For example, the siRNA of the invention can

be delivered to retinal pigment epithelial cells in the eye. Preferably, the siRNA is administered topically to the eye, *e.g.* in liquid or gel form to the lower eye lid or conjunctival cul-de-sac, as is within the skill in the art (see, *e.g.*, Acheampong AA et al, 2002, *Drug Metabol. and Disposition* 30: 421-429, the entire disclosure of which 5 is herein incorporated by reference).

Typically, the siRNA of the invention is administered topically to the eye in volumes of from about 5 microliters to about 75 microliters, for example from about 7 microliters to about 50 microliters, preferably from about 10 microliters to about 30 microliters. The siRNA of the invention is highly soluble in aqueous solutions, It is 10 understood that topical instillation in the eye of siRNA in volumes greater than 75 microliters can result in loss of siRNA from the eye through spillage and drainage. Thus, it is preferable to administer a high concentration of siRNA (*e.g.*, 100-1000 nM) by topical instillation to the eye in volumes of from about 5 microliters to about 75 microliters.

15 A particularly preferred parenteral administration route is intraocular administration. It is understood that intraocular administration of the present siRNA can be accomplished by injection or direct (*e.g.*, topical) administration to the eye, as long as the administration route allows the siRNA to enter the eye. In addition to the topical routes of administration to the eye described above, suitable intraocular routes 20 of administration include intravitreal, intraretinal, subretinal, subtenon, peri- and retro-orbital, trans-corneal and trans-scleral administration. Such intraocular administration routes are within the skill in the art; see, *e.g.*, and Acheampong AA et al, 2002, *supra*; and Bennett et al. (1996), *Hum. Gene Ther.* 7: 1763-1769 and Ambati J et al., 2002, *Progress in Retinal and Eye Res.* 21: 145-151, the entire disclosures of 25 which are herein incorporated by reference.

The siRNA of the invention can be administered in a single dose or in multiple doses. Where the administration of the siRNA of the invention is by infusion, the infusion can be a single sustained dose or can be delivered by multiple infusions. 30 Injection of the siRNA directly into the tissue is at or near the site of neovascularization preferred. Multiple injections of the siRNA into the tissue at or near the site of neovascularization are particularly preferred.

One skilled in the art can also readily determine an appropriate dosage regimen for administering the siRNA of the invention to a given subject. For example, the siRNA can be administered to the subject once, such as by a single injection or deposition at or near the neovascularization site. Alternatively, the siRNA can be 5 administered to a subject multiple times daily or weekly. For example, the siRNA can be administered to a subject once weekly for a period of from about three to about twenty-eight weeks, more preferably from about seven to about ten weeks. In a preferred dosage regimen, the siRNA is injected at or near the site of neovascularization (e.g., intravitreally) once a week for seven weeks. It is understood that periodic 10 administrations of the siRNA of the invention for an indefinite length of time may be necessary for subjects suffering from a chronic neovascularization disease, such as wet ARMD or diabetic retinopathy.

Where a dosage regimen comprises multiple administrations, it is understood that the effective amount of siRNA administered to the subject can comprise the total 15 amount of siRNA administered over the entire dosage regimen.

The siRNA of the invention are preferably formulated as pharmaceutical compositions prior to administering to a subject, according to techniques known in the art. Pharmaceutical compositions of the present invention are characterized as being at least sterile and pyrogen-free. As used herein, "pharmaceutical formulations" 20 include formulations for human and veterinary use. Methods for preparing pharmaceutical compositions of the invention are within the skill in the art, for example as described in *Remington's Pharmaceutical Science*, 17th ed., Mack Publishing Company, Easton, Pa. (1985), the entire disclosure of which is herein incorporated by reference.

25 The present pharmaceutical formulations comprise an siRNA of the invention (e.g., 0.1 to 90% by weight), or a physiologically acceptable salt thereof, mixed with a physiologically acceptable carrier medium. Preferred physiologically acceptable carrier media are water, buffered water, saline solutions (e.g., normal saline or balanced saline solutions such as Hank's or Earle's balanced salt solutions), 0.4% 30 saline, 0.3% glycine, hyaluronic acid and the like.

Pharmaceutical compositions of the invention can also comprise conventional pharmaceutical excipients and/or additives. Suitable pharmaceutical excipients

include stabilizers, antioxidants, osmolality adjusting agents, buffers, and pH adjusting agents. Suitable additives include physiologically biocompatible buffers (e.g., tromethamine hydrochloride), additions of chelants (such as, for example, DTPA or DTPA-bisamide) or calcium chelate complexes (as for example calcium 5 DTPA, CaNaDTPA-bisamide), or, optionally, additions of calcium or sodium salts (for example, calcium chloride, calcium ascorbate, calcium gluconate or calcium lactate). Pharmaceutical compositions of the invention can be packaged for use in liquid form, or can be lyophilized.

For topical administration to the eye, conventional intraocular delivery 10 reagents can be used. For example, pharmaceutical compositions of the invention for topical intraocular delivery can comprise saline solutions as described above, corneal penetration enhancers, insoluble particles, petrolatum or other gel-based ointments, polymers which undergo a viscosity increase upon instillation in the eye, or mucoadhesive polymers. Preferably, the intraocular delivery reagent increases 15 corneal penetration, or prolongs preocular retention of the siRNA through viscosity effects or by establishing physicochemical interactions with the mucin layer covering the corneal epithelium.

Suitable insoluble particles for topical intraocular delivery include the calcium 20 phosphate particles described in U.S. Pat. No. 6,355,271 of Bell et al., the entire disclosure of which is herein incorporated by reference. Suitable polymers which undergo a viscosity increase upon instillation in the eye include polyethylenepolyoxypropylene block copolymers such as poloxamer 407 (e.g., at a concentration of 25%), cellulose acetophthalate (e.g., at a concentration of 30%), or a low-acetyl gellan gum such as Gelrite® (available from CP Kelco, Wilmington, DE). 25 Suitable mucoadhesive polymers include hydrocolloids with multiple hydrophilic functional groups such as carboxyl, hydroxyl, amide and/or sulfate groups; for example, hydroxypropylcellulose, polyacrylic acid, high-molecular weight polyethylene glycols (e.g., >200,000 number average molecular weight), dextrans, hyaluronic acid, polygalacturonic acid, and xylocan. Suitable corneal penetration 30 enhancers include cyclodextrins, benzalkonium chloride, polyoxyethylene glycol lauryl ether (e.g., Brij® 35), polyoxyethylene glycol stearyl ether (e.g., Brij® 78), polyoxyethylene glycol oleyl ether (e.g., Brij® 98), ethylene diamine tetraacetic acid

(EDTA), digitonin, sodium taurocholate, saponins and polyoxyethylated castor oil such as Cremaphor EL.

For solid compositions, conventional nontoxic solid carriers can be used; for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, 5 sodium saccharin, talcum, cellulose, glucose, sucrose, magnesium carbonate, and the like.

For example, a solid pharmaceutical composition for oral administration can comprise any of the carriers and excipients listed above and 10-95%, preferably 25%-75%, of one or more siRNA of the invention. A pharmaceutical composition for 10 aerosol (inhalational) administration can comprise 0.01-20% by weight, preferably 1%-10% by weight, of one or more siRNA of the invention encapsulated in a liposome as described above, and propellant. A carrier can also be included as desired; *e.g.*, lecithin for intranasal delivery.

15 The invention will now be illustrated with the following non-limiting examples. The animal experiments described below were performed using the University of Pennsylvania institutional guidelines for the care and use of animals in research.

20 **Example 1 – Inhibition of Human VEGF Expression in Cultured Human Embryonic Kidney Cells with Anti-HIF-1 Alpha siRNAs**

Human embryonic kidney 293 (HEK-293) cells were cultured in 24 well plates at 37°C with 5% CO₂ overnight, in standard growth medium. Transfections 25 were performed the following day on experimental and control cells, when the cells were approximately 50% confluent. The experimental cells were transfected with 25 nM human HIF-1 alpha siRNA mixed in calcium phosphate reagent. Control cells were treated with transfection reagent lacking siRNA, or with 25 nM nonspecific siRNA (EGFP1 siRNA) in calcium phosphate transfection reagent. For the 30 experimental cells, twenty different siRNAs targeted to human HIF-1 alpha mRNA were tested. These anti-HIF-1 alpha siRNAs contained the targeting sequences listed in Table 2, and all siRNAs contained 3' TT overhangs on each strand. The "sample

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#" listed in Table 2 corresponds to the experimental cell sample as indicated in Figs. 1 and 2.

Table 2 – Target Sequences for Anti-HIF-1 Alpha siRNAs Tested in HEK-293 Cells

Target Sequence	SEQ ID NO:	Sample #
AACTAGCCGAGGAAGAACTAT	76	1
AACTGTCATATATAACACCAA	117	2
AATTACGTTGTGAGTGGTATT	122	3
AAACGCCAAAGCCACTTCGAA	161	4
AAAGTTCACCTGAGCCTAATA	177	5
AAGTTCACCTGAGCCTAATAG	180	6
AAAGCACAGTTACAGTATTCC	200	7
AAGCACAGTTACAGTATTCCA	201	8
AAAAGACCGTATGGAAGACAT	212	9
AACTACTAGTGCCACATCATC	222	10
AAAGTCGGACAGCCTCACCAA	223	11
AAGTCGGACAGCCTCACCAA	224	12
AACGTGTTATCTGTCGCTTG	237	13
AAGCAGTAGGAATTGGAACAT	255	14
AATGGATGAAAGTGGATTACC	274	15
AATGTGAGTTCGCATTTGAT	40	16
AAGATGACATGAAAGCACAGA	44	17
AACTGGACACAGTGTGTTGA	56	18
AAATTCCCTTAGATAGCAAGA	93	19
AAACCGGTTGAATCTTCAGAT	127	20

5 At four hours post-transfection, hypoxia was induced in control and experimental HEK-293 cells with desferrioxamine at a final concentration of 200 micromolar. At 48 hours post transfection, the cell culture medium was removed from all wells and a human VEGF ELISA (R & D systems, Minneapolis, MN) was performed as described in the Quantikine human VEGF ELISA protocol. ELISA 10 results were read on an AD340 plate reader (Beckman Coulter), and are given in Fig. 1.

As can be seen from Fig. 1, human VEGF protein was upregulated in HEK-293 cells by the desferrioxamine-mediated induction of hypoxia. The hypoxia-induced increase in VEGF protein was reduced in cells transfected with the human anti-HIF-1 alpha siRNAs. Transfections of hypoxic cells with non-specific siRNA 5 (EGFP siRNA) or mock transfection without siRNA had no effect on VEGF protein levels. The anti-HIF-1 alpha siRNAs hHIF1#12, hHIF1#13 and hHIF1#16 reduced VEGF protein expression to levels approaching that of non-hypoxic HEK-293 cells. Anti-HIF-1 alpha siRNA hHIF1#11 reduced VEGF protein expression to below that of non-hypoxic HEK-293 cells.

10 After the cell culture medium was removed from the control and experimental cells, a cytotoxicity assay was performed as follows. Complete growth medium containing 10% AlamarBlue (Biosource, Camarillo, CA) was added to each well, and the cells were incubated at 37°C with 5% CO₂ for 3 hours. Cell proliferation was measured by detecting the color change of medium containing AlamarBlue resulting 15 from cell metabolic activity. Cytotoxicity assay results were read on an AD340 plate reader (Beckman Coulter) and are given in Fig. 2. As can be seen from Fig. 2, none of the twenty anti-HIF-1 alpha siRNAs tested showed significant cytotoxicity in the HEK-293 cells.

After the cytotoxicity assay was performed, the growth medium in each well 20 was completely removed, and RNA extractions from the HEK-293 cells were performed with the RNAqueous RNA isolation kit (Ambion, Austin, TX) according to the manufacturer's instructions. The levels of human HIF-1 alpha and VEGF mRNA in the cells were measured by quantitative reverse transcription-polymerase chain reaction (RT-PCR), using the level of human glyceraldehyde-3-phosphate 25 dehydrogenase (GAPDH) mRNA as an internal standard.

The RT-PCR study showed that hypoxia increased the mRNA levels of human VEGF relative to VEGF mRNA expression in non-hypoxic cells. The VEGF mRNA levels in hypoxic cells were reduced by transfection with anti-HIF-1 alpha siRNAs. Transfection of hypoxic cells with non-specific siRNA (EGFP siRNA) or mock 30 transfection with no siRNA did not reduce VEGF mRNA levels. Thus, the introduction of anti-HIF-1 alpha siRNAs into the HEK-293 cells induced the destruction of the VEGF mRNA, as compared to cells transfected with non-specific

siRNA or no siRNA. The destruction of VEGF mRNA induced by the anti-HIF-1 alpha siRNAs correlated with the reduction in VEGF protein production shown in Fig. 1.

5 **Example 2 – In Vivo Inhibition of Angiogenesis with Anti-HIF-1 Alpha siRNA in a Mouse Model of Choroidal Neovascularization**

Adult (8-15 week old) female C57Bl/6 mice (n=7) were anesthetized with avertin (2,2,2-tribromoethanol) and their pupils were dilated with 1% tropicamide. 10 Laser photocoagulation was performed bilaterally using a diode laser photocoagulator (IRIS Medical, Mountain View, CA) and a slit lamp system with a cover slip as a contact lens. Laser photocoagulation (140 mW; 75 micron spot size; 0.1 s duration) was applied to the 9, 12 and 3 o'clock positions in both eyes at 2 to 3 disk diameters from the optic nerve. Since the rupture of Bruch's membrane is necessary to create 15 significant choroidal neovascularization (CNV), bubble formation at the time of photocoagulation was used as an indication of the rupture of Bruch's membrane. Laser burns that did not induce a rupture in Bruch's membrane were excluded from the study.

Immediately after laser treatment, an siRNA targeted to mouse HIF-1 alpha 20 mRNA was delivered to both eyes of each animal in the test group by intravitreal injection. Control animals received intravitreal injection of carrier only.

The target sequence of the mouse anti-HIF-1 alpha mRNA was AACTAACTGGACACAGTGTGT (SEQ ID NO: 297), and the siRNA used was:

25 5'-cuaacuggacacaguguguTT-3' (SEQ ID NO: 298)
3'-TTgauugaccugugucacaca-5' (SEQ ID NO: 299)

Twelve days after laser photocoagulation, the animals were perfused with high molecular weight dextran-fluorescein (Molecular Probes, Eugene, OR) to label the retinal/choroidal vasculature, and the eyes were harvested. The area of each CNV was measured in choroidal flat mount preparations.

30 To prepare choroidal flat mounts, the anterior chamber was removed and the retina was extracted with the vitreous, leaving the eyecup. Relaxing incisions were made on the eye cup and the choroid was flattened onto a slide. Using a Leica DMR microscope (Wetzlar, Germany) equipped with epifluorescence illumination, a

masked investigator identified lesions in the dextran-fluorescein-perfused flat mount preparations as circular fluorescent (fluorescein positive) areas corresponding to the area previously exposed to the laser light. Images of the lesions were captured using a black and white Hamamatsu CCD camera (Hamamatsu Photonics, Bridgewater, NJ) 5 coupled to a Apple Macintosh G4 computer (Cupertino, CA) equipped with OpenLab 2.2 software. Images for calibration were obtained from a slide with a grating of known size. The hyperfluorescent fluorescein-dextran labeled blood vessels within the area of the laser burn were selected as "region of interest" (ROI) using Openlab software, and this software was used to calculate the area (μm^2) occupied by the white 10 pixels in the ROIs. The ROIs were selected after collecting the images under identical integration settings by using the Openlab "magic wand" tool to identify pixels in the laser burn site at a range of 2000-4090 intensity units, as defined within the Openlab software. The intensity units which were selected represented levels measured in normal fluorescein-perfused vasculature. For reference, the intensity of background, 15 non-fluorescent areas was <450 intensity units.

The ROIs were generally well-circumscribed by a region lacking fluorescence. After measuring the areas of CNV, images were colorized in Openlab by applying an intensity ramp at 515 nanometers (the wavelength at which the image data were captured), using the "apply wavelength" function in the Openlab software. This 20 intensity ramp was applied to all of the pixels in the image, and made the whitest pixels the brightest green color. The images were then exported to Adobe Photoshop software for presentation purposes. Situations in which there was no evidence of a laser burn after bright field analysis of choroidal flatmounts were excluded.

Statistical analysis of the results was performed using a one-tailed distribution, 25 two sample unequal variance Student's t-test. There was a statistically significant reduction in the CNV area ($P = 0.000354$) between the anti-HIF-1 alpha siRNA treated animals and the control lasered animals, indicating a substantial reduction in angiogenesis in the animals receiving the anti-HIF-1 alpha siRNA. The results are presented in Fig. 3.

We claim:

1. An isolated siRNA comprising a sense RNA strand and an antisense RNA strand, wherein the sense and an antisense RNA strands form an RNA duplex, and wherein the sense RNA strand comprises a nucleotide sequence substantially identical to a target sequence of about 19 to about 25 contiguous nucleotides in human HIF-1 alpha mRNA, or an alternative splice form, mutant or cognate thereof.
- 10 2. The siRNA of claim 1, wherein the human HIF-1 alpha mRNA is SEQ ID NO: 1.
- 15 3. The siRNA of claim 1, wherein the cognate of the human HIF-1 alpha mRNA sequence is rat HIF-1 alpha mRNA or mouse HIF-1 alpha mRNA.
4. The siRNA of claim 1, wherein the sense RNA strand comprises one RNA molecule, and the antisense RNA strand comprises one RNA molecule.
- 20 5. The siRNA of claim 1, wherein the sense and antisense RNA strands forming the RNA duplex are covalently linked by a single-stranded hairpin.
6. The siRNA of claim 1, wherein the siRNA further comprises non-nucleotide material.
- 25 7. The siRNA of claim 1, wherein the siRNA further comprises an addition, deletion, substitution or alteration of one or more nucleotides.
8. The siRNA of claim 1, wherein the sense and antisense RNA strands are stabilized against nuclease degradation.
- 30 9. The siRNA of claim 1, further comprising a 3' overhang.

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10. The siRNA of claim 9, wherein the 3' overhang comprises from 1 to about 6 nucleotides.

11. The siRNA of claim 9, wherein the 3' overhang comprises about 2
5 nucleotides.

12. The siRNA of claim 5, wherein the sense RNA strand comprises a first 3' overhang, and the antisense RNA strand comprises a second 3' overhang.

10 13. The siRNA of claim 12, wherein the first and second 3' overhangs separately comprise from 1 to about 6 nucleotides.

14. The siRNA of claim 13, wherein the first 3' overhang comprises a dinucleotide and the second 3' overhang comprises a dinucleotide.

15 15. The siRNA of claim 14, where the dinucleotide comprising the first and second 3' overhangs is dithymidyl acid (TT) or diuridylic acid (uu).

16. The siRNA of claim 9, wherein the 3' overhang is stabilized against
20 nuclease degradation.

17. A retinal pigment epithelial cell comprising the siRNA of claim 1.

18. A recombinant plasmid comprising nucleic acid sequences for
25 expressing an siRNA comprising a sense RNA strand and an antisense RNA strand, wherein the sense and an antisense RNA strands form an RNA duplex, and wherein the sense RNA strand comprises a nucleotide sequence substantially identical to a target sequence of about 19 to about 25 contiguous nucleotides in human HIF-1 alpha mRNA, or an alternative splice form, mutant or cognate thereof.

30 19. The recombinant plasmid of claim 18, wherein the nucleic acid sequences for expressing the siRNA comprise an inducible or regulatable promoter.

20. The recombinant plasmid of claim 18, wherein the nucleic acid sequences for expressing the siRNA comprise a sense RNA strand coding sequence in operable connection with a polyT termination sequence under the control of a human
5 U6 RNA promoter, and an antisense RNA strand coding sequence in operable connection with a polyT termination sequence under the control of a human U6 RNA promoter.

21. The recombinant plasmid of claim 20, wherein the plasmid is
10 pAAVsiRNA.

22. A recombinant viral vector comprising nucleic acid sequences for expressing an siRNA comprising a sense RNA strand and an antisense RNA strand, wherein the sense and an antisense RNA strands form an RNA duplex, and wherein
15 the sense RNA strand comprises a nucleotide sequence substantially identical to a target sequence of about 19 to about 25 contiguous nucleotides in human HIF-1 alpha mRNA, or an alternative splice form, mutant or cognate thereof.

23. The recombinant viral vector of claim 22, wherein the nucleic acid
20 sequences for expressing the siRNA comprise an inducible or regulatable promoter.

24. The recombinant viral vector of claim 22, wherein the nucleic acid sequences for expressing the siRNA comprise a sense RNA strand coding sequence in operable connection with a polyT termination sequence under the control of a human
25 U6 RNA promoter, and an antisense RNA strand coding sequence in operable connection with a polyT termination sequence under the control of a human U6 RNA promoter.

25. The recombinant viral vector of claim 22, wherein the recombinant
30 viral vector is selected from the group consisting of an adenoviral vector, an adeno-associated viral vector, a lentiviral vector, a retroviral vector, and a herpes virus vector.

26. The recombinant viral vector of claim 22, wherein the recombinant viral vector is pseudotyped with surface proteins from vesicular stomatitis virus, rabies virus, Ebola virus, or Mokola virus.

5

27. The recombinant viral vector of claim 25, wherein the recombinant viral vector comprises an adeno-associated viral vector.

28. A pharmaceutical composition comprising an siRNA and a pharmaceutically acceptable carrier, wherein the siRNA comprises a sense RNA strand and an antisense RNA strand, wherein the sense and an antisense RNA strands form an RNA duplex, and wherein the sense RNA strand comprises a nucleotide sequence substantially identical to a target sequence of about 19 to about 25 contiguous nucleotides in human HIF-1 alpha mRNA, or an alternative splice form, mutant or cognate thereof.

10 29. The pharmaceutical composition of claim 28, further comprising lipofectin, lipofectamine, cellfectin, polycations, or liposomes.

15 30. A pharmaceutical composition comprising the plasmid of claim 18, or a physiologically acceptable salt thereof, and a pharmaceutically acceptable carrier.

20 31. The pharmaceutical composition of claim 30, further comprising lipofectin, lipofectamine, cellfectin, polycations, or liposomes.

25 32. A pharmaceutical composition comprising the viral vector of claim 22 and a pharmaceutically acceptable carrier.

30 33. A method of inhibiting expression of human HIF-1 alpha mRNA, or an alternative splice form, mutant or cognate thereof, comprising administering to a subject an effective amount of an siRNA comprising a sense RNA strand and an antisense RNA strand, wherein the sense and an antisense RNA strands form an RNA

duplex, and wherein the sense RNA strand comprises a nucleotide sequence substantially identical to a target sequence of about 19 to about 25 contiguous nucleotides in human HIF-1 alpha mRNA, or an alternative splice form, mutant or cognate thereof, such that human HIF-1 alpha mRNA, or an alternative splice form, 5 mutant or cognate thereof, is degraded.

34. The method of claim 33, wherein the subject is a human being.

35. The method of claim 33, wherein expression of human HIF-1 alpha 10 mRNA, or an alternative splice form, mutant or cognate thereof is inhibited in one or both eyes of the subject.

36. The method of claim 33, wherein expression of human HIF-1 alpha mRNA, or an alternative splice form, mutant or cognate thereof is inhibited in retinal 15 pigment epithelial cells of the subject.

37. The method of claim 33, wherein the effective amount of the siRNA is an amount which provides an intercellular concentration at or near the neovascularization site of from about 1 nM to about 100 nM.

20

38. The method of claim 33, wherein the siRNA is administered in conjunction with a delivery reagent.

39. The method of claim 38, wherein the delivery agent is selected from 25 the group consisting of lipofectin, lipofectamine, cellfectin, polycations, and liposomes.

40. The method of claim 39, wherein the delivery agent is a liposome.

30 41. The method of claim 40, wherein the liposome comprises a ligand which targets the liposome to cells at or near the site of angiogenesis.

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42. The method of claim 41, wherein the ligand binds to receptors on tumor cells or vascular endothelial cells.

43. The method of claim 42, wherein the ligand comprises a monoclonal 5 antibody.

44. The method of claim 40, wherein the liposome is modified with an opsonization-inhibition moiety.

10 45. The method of claim 44, wherein the opsonization-inhibiting moiety comprises a PEG, PPG, or derivatives thereof.

46. The method of claim 33, wherein the siRNA is expressed from a recombinant plasmid

15 47. The method of claim 33, wherein the siRNA is expressed from a recombinant viral vector.

20 48. The method of claim 47, wherein the recombinant viral vector comprises an adenoviral vector, an adeno-associated viral vector, a lentiviral vector, a retroviral vector, or a herpes virus vector.

49. The method of claim 48, wherein the recombinant viral vector is pseudotyped with surface proteins from vesicular stomatitis virus, rabies virus, Ebola 25 virus, or Mokola virus.

50. The method of claim 47, wherein the recombinant viral vector comprises an adeno-associated viral vector.

30 51. The method of claim 33, wherein the siRNA is administered by an enteral administration route.

52. The method of claim 51, wherein the enteral administration route is selected from the group consisting of oral, rectal, and intranasal.

53. The method of claim 33, wherein the siRNA is administered by a 5 parenteral administration route.

54. The method of claim 53, wherein the parenteral administration route is selected from the group consisting of intravascular administration, peri- and intra-tissue administration, subcutaneous injection or deposition, subcutaneous infusion, 10 intraocular administration, and direct application at or near the site of neovascularization.

55. The method of claim 54, wherein the intravascular administration is selected from the group consisting of intravenous bolus injection, intravenous 15 infusion, intra-arterial bolus injection, intra-arterial infusion and catheter instillation into the vasculature.

56. The method of claim 54, wherein the peri- and intra-tissue injection comprises peri-tumoral injection or intra-tumoral injection.

20

57. The method of claim 54, wherein the intraocular administration comprises intravitreal, intraretinal, subretinal, subtenon, peri- and retro-orbital, trans-corneal or trans-scleral administration.

25

58. The method of claim 54, wherein the direct application at or near the site of neovascularization comprises application by catheter, corneal pellet, eye dropper, suppository, an implant comprising a porous material, an implant comprising a non-porous material, or an implant comprising a gelatinous material.

30

59. The method of claim 54, wherein the site of neovascularization is in the eye, and the direct application at or near the site of neovascularization comprises application by an ocular implant.

60. The method of claim 59, wherein the ocular implant is biodegradable.

61. A method of inhibiting angiogenesis in a subject, comprising
5 administering to a subject an effective amount of an siRNA comprising a sense RNA
strand and an antisense RNA strand, wherein the sense and an antisense RNA strands
form an RNA duplex, and wherein the sense RNA strand comprises a nucleotide
sequence substantially identical to a target sequence of about 19 to about 25
contiguous nucleotides in human HIF-1 alpha mRNA, or an alternative splice form,
10 mutant or cognate thereof.

62. The method of claim 61, wherein the angiogenesis is pathogenic.

63. The method of claim 61, wherein the angiogenesis is non-pathogenic.
15

64. The method of claim 63, wherein the non-pathogenic angiogenesis is
associated with production of fatty tissues or cholesterol production.

65. The method of claim 63, wherein the non-pathogenic angiogenesis
20 comprises endometrial neovascularization.

66. The method of claim 61, wherein the angiogenesis is inhibited in one
or both eyes of the subject.

67. A method of treating an angiogenic disease in a subject, comprising
25 administering to a subject an effective amount of an siRNA comprising a sense RNA
strand and an antisense RNA strand, wherein the sense and an antisense RNA strands
form an RNA duplex, and wherein the sense RNA strand comprises a nucleotide
sequence substantially identical to a target sequence of about 19 to about 25
30 contiguous nucleotides in human HIF-1 alpha mRNA, or an alternative splice form,
mutant or cognate thereof, such that angiogenesis associated with the angiogenic
disease is inhibited.

68. The method of claim 67, wherein the angiogenic disease comprises a tumor associated with a cancer.

5 69. The method of claim 68, wherein the cancer is selected from the group consisting of breast cancer, lung cancer, head and neck cancer, brain cancer, abdominal cancer, colon cancer, colorectal cancer, esophagus cancer, gastrointestinal cancer, glioma, liver cancer, tongue cancer, neuroblastoma, osteosarcoma, ovarian cancer, pancreatic cancer, prostate cancer, retinoblastoma, Wilm's tumor, multiple
10 myeloma, skin cancer, lymphoma, and blood cancer.

70. The method of claim 67, wherein the angiogenic disease is selected from the group consisting of diabetic retinopathy, age-related macular degeneration, and inflammatory diseases.

15 71. The method of claim 70, wherein the inflammatory disease is psoriasis or rheumatoid arthritis.

72. The method of claim 70, wherein the angiogenic disease is age-related
20 macular degeneration.

73. The method of claim 67, wherein the siRNA is administered in combination with a pharmaceutical agent for treating the angiogenic disease, which pharmaceutical agent is different from the siRNA.

25 74. The method of claim 73, wherein the angiogenic disease is cancer, and the pharmaceutical agent comprises a chemotherapeutic agent.

75. The method of claim 73, wherein the chemotherapeutic agent is
30 selected from the group consisting of cisplatin, carboplatin, cyclophosphamide, 5-fluorouracil, adriamycin, daunorubicin, and tamoxifen.

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76. The method of claim 67, wherein the siRNA is administered to a subject in combination with another therapeutic method designed to treat the angiogenic disease.

5 77. The method of claim 76, wherein the angiogenic disease is cancer, and the siRNA is administered in combination with radiation therapy, chemotherapy or surgery.

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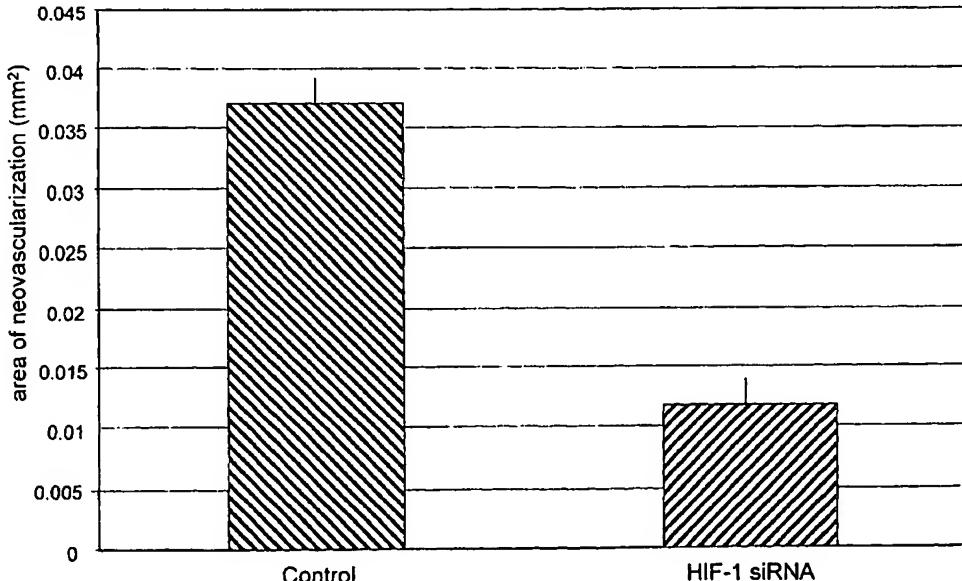
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(54) Title: COMPOSITIONS AND METHODS FOR siRNA INHIBITION OF HIF-1 ALPHA



(57) Abstract: RNA interference using small interfering RNAs which target HIF-1 alpha mRNA inhibit expression of the HIF-1 alpha gene. As HIF-1 alpha is a transcriptional regulator of VEGF, expression of VEGF is also inhibited. Control of VEGF production through siRNA-mediated down-regulation of HIF-1 alpha can be used to inhibit angiogenesis, in particularly in diseases such as diabetic retinopathy, age related macular degeneration and many types of cancer.

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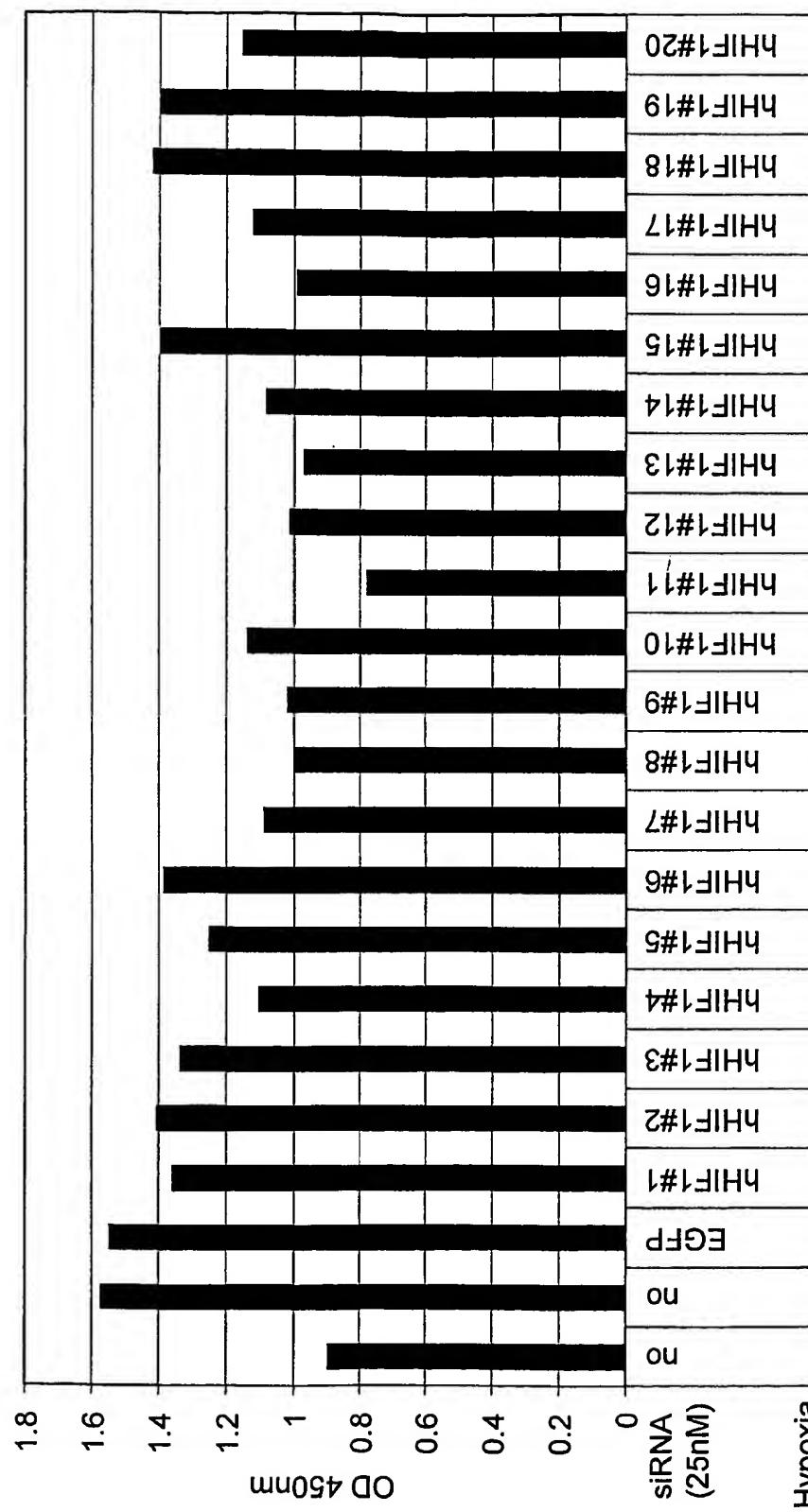


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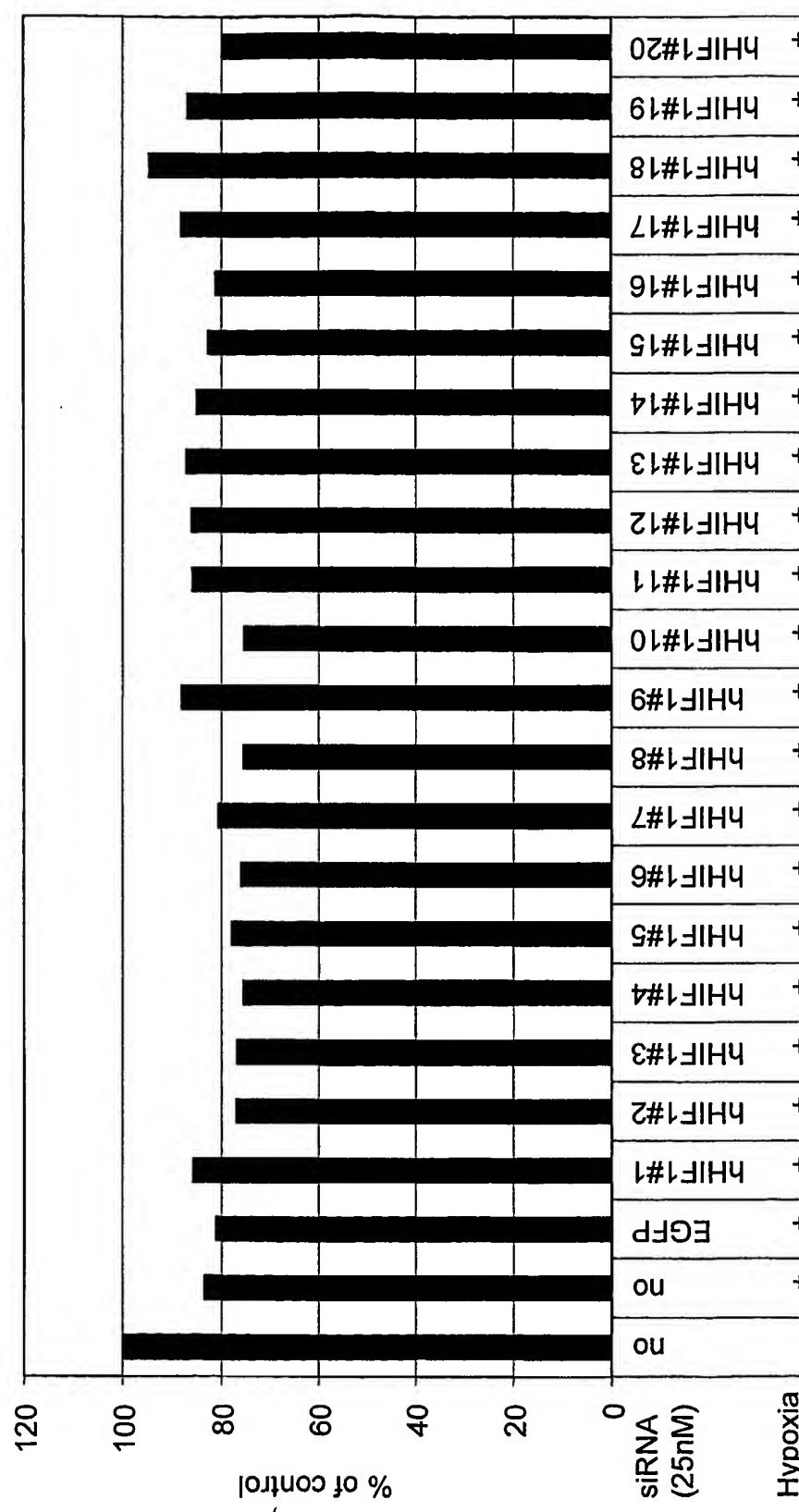


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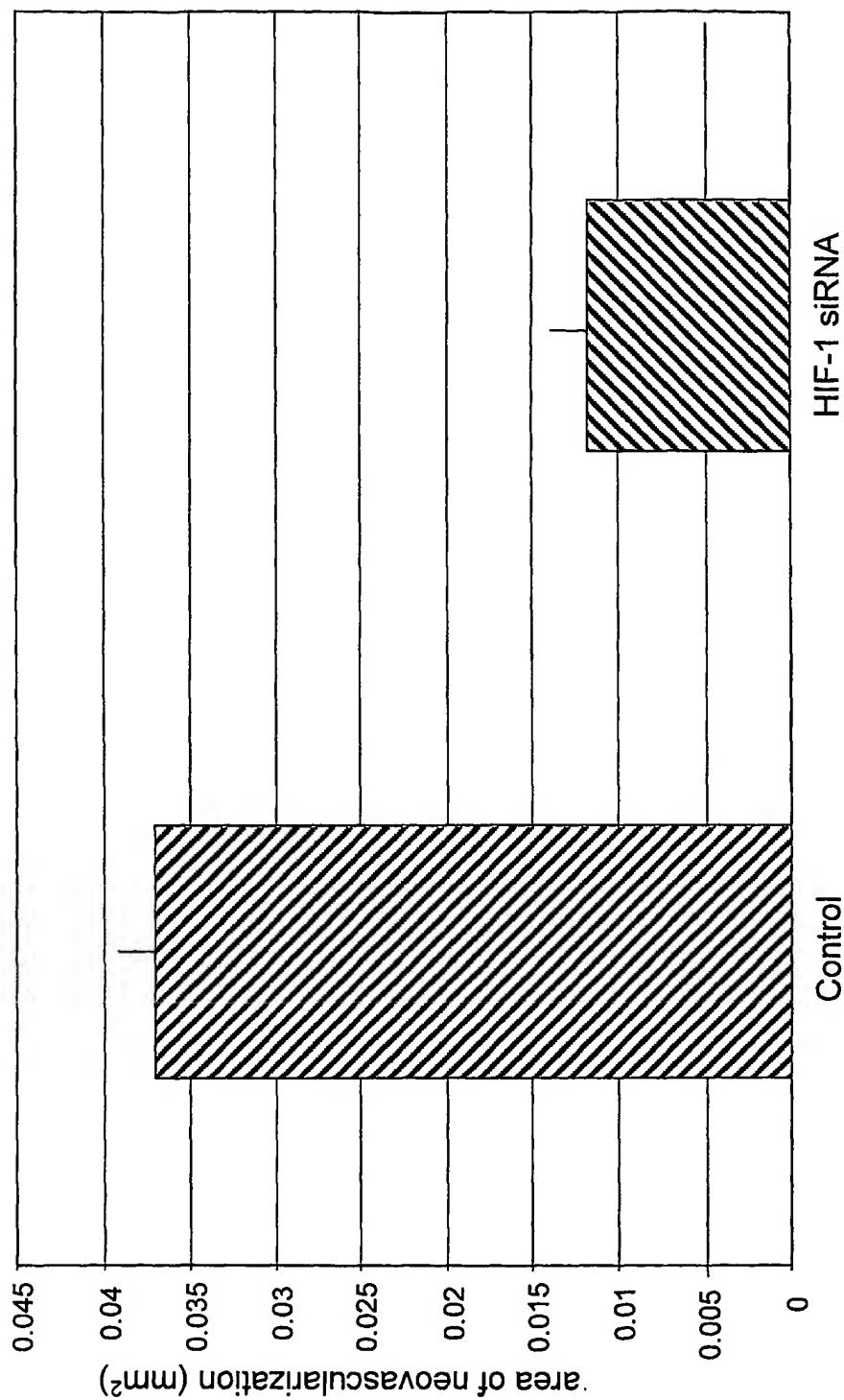


Fig. 3

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US03/34826

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C07H 21/04; A61K 48/00; C12N 15/00
 US CL : 435/375; 536/24.5; 514/44

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
 U.S. : 435/375; 536/24.5; 514/44

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
 WEST, MEDLINE, BIOSIS, CAPLUS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SUN et al. Gene Transfer of Antisense Hypoxia Inducible Factor-1 alpha Enhances the Therapeutic Efficacy of Cancer Immunotherapy. Gene Therapy. 2001, Vol. 8, pages 638-645, see entire document.	1-77
Y	KANG et al. An Antisense Oligonucleotide That Inhibits the Expression of Hypoxia-Inducible Factor-1 alpha Alters Hypoxia-Induced Changes in Proliferation and Viability of Human Cardiac Fibroblasts., page II.57, see abstract.	1-77
Y,P	HOEG et al. In vitro and in vivo Efficacy of a HIF-1alpha-Antisense Oligonucleotide Containing Locked Nucleic Acids. ECJ Supplements. September 2003, pages S212-S213, see entire abstract.	1-77

<input type="checkbox"/>	Further documents are listed in the continuation of Box C.	<input type="checkbox"/>	See patent family annex.
*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"B"	earlier application or patent published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 03 May 2004 (03.05.2004)	Date of mailing of the international search report 07 JUL 2004
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US Commissioner for Patents P.O. Box 1450 Alexandria, Virginia 22313-1450 Facsimile No. (703) 305-3230	Authorized officer Sean R McGarry Telephone No. 703 308-0196 <i>Janice Ford</i> J. Ford